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Laser-induced, photooxidative damage in ocular tissue was studied with a quantitative assay using 1 performance liquid chromatography (HPLC) to separate oxidized and reduced ascorbic acid in exposed ti components. We demonstrated that ascorbic acid, incubated with whole, bovine retinal pigment epithelial (R cells, was oxidized when the reaction mixture was exposed to the output of an argon-ion continuous wave l: The amount of ascorbic acid oxidized was proportional to the irradiance of the sample, and the reaction wavelength-dependent, with short-wavelength visible light more effective than long-wavelengths in driving reaction. The photosensitizing activity was associated with the RPE melanin pigment granules, and was not after disrupting or heating the RPE cells. Because melanin was known to form free radicals when illuminated hypothesized that ascorbic acid detoxified the light-activated melanin free radicals while being itself oxidized in process. If the supply of reduced ascorbic acid were exhausted, however, the activated melanin could have be source of tissue-damaging radicals. This model was consistent with a photochemical damage mechat involving light-activated melanin.

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REDUCTION OF PHOTOOXIDATIVE STRESS IN THE RETINA BY UPTAKE OF DEHYDRO-L-ASCORBIC ACID

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We recently reported that light-activated, RPE melanin oxidizes ascorbic aci (AA) to an extent proportional to the radiant exposure (Glickman & Lam Photochem Photobiol, 55:191-196, 1992). It is known that light excites melanin t an active free-radical state. Because AA is present at up to 1 mM in ocular tissue: we hypothesized that AA donates electrons to light-activated melanin. We furthe reasoned that if the supply of reduced AA were to be exhausted, then the melani radicals could react with cellular structures, causing damage and loss of function

The use of AA to prevent photic damage to the retina has been explored in ou laboratory and by others. We compared the transport kinetics of reduced AA t those of its oxidized form, dehydro-L-ascorbic acid (DHA), in cultured, trans-forme RPE cells. AA is transported into RPE cells by a high-affinity (K_m=0.041 mM mechanism with a low V_{max} (2.74 pmol/min), whereas the K_m of the DH, transporter is 5.670 mM, and its V_{max} is 325 pmol/min. The level of AA in serur normally saturates the AA transport system; therefore, administration of AA doe not result in increased transport of AA into tissues. DHA, however, is normally lo in the serum, and thus is actively transported into RPE cells where it is rapidl reduced to AA. We have demonstrated, by recording the ERG from a superfuse rabbit eyecup preparation, that addition of DHA, but not AA, at 1 mM or highe protects the retina against photic damage. This result indicates that DHA may t used therapeutically to increase the content of AA in sensitive ocular tissue

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OXIDATION OF ASCORBIC ACID AS AN INDICATOR
OF PHOTOOXIDATIVE STRESS IN THE EYE

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Abstract—When whole retinal pigmented epithelium (RPE) cells isolated from bovine eyes are incubated with ^{14}C-labeled ascorbic acid and exposed to a visible laser, the ascorbic acid is oxidized to dehydro-L-ascorbic acid (DHA). The amount of ascorbic acid which is oxidized is proportional to the radiant exposure of the sample (*i.e.* the total amount of radiation per unit area delivered over the exposure time). Blue light is more effective than red light in driving the reaction. The amount of label appearing in the DHA fraction is increased if unlabeled DHA is present in the reaction mixture, indicating that some redox cycling of ascorbate is occurring in the RPE cells. The ascorbic acid oxidizing activity does not depend on intact cells, is not inactivated by heating the cells to 80°C, and appears to reside mainly in the subcellular fraction which contains melanin pigment granules. The ascorbic acid oxidation may be caused by free radicals formed when melanin is illuminated with light. This reaction appears to be a useful method for quantifying the production of free radicals during photooxidative stress.

INTRODUCTION

Previous observations have found that the ascorbic acid (AA)† in the retina, aqueous humor, and vitreous exists primarily in the reduced form, while in the retinal pigmented epithelium (RPE) and choroid, the oxidized form, dehydro-L-ascorbic acid (DHA), is relatively more plentiful (Woodford *et al.*, 1983; Lai *et al.*, 1986). Because of the ability of AA to undergo oxidation-reduction cycling, it is able to serve as a physiological antioxidant and free radical scavenger (Sapper *et al.*, 1982; Bielski and Richter, 1975). Thus, the oxidation of AA may serve as a possible marker of oxidative stress in biological tissues. Indeed, when retinal damage is induced by chronic light exposure, the amount of DHA markedly increases in the RPE (Woodford *et al.*, 1983; Tso *et al.*, 1984; Fong *et al.*, 1987). The apparent oxidation of AA during intense light exposure suggests that it is serving as a substrate for oxidizing species produced by the interaction of light with a sensitizer in the ocular tissues. We designed this series of experiments to determine if the AA in ocular tissues could be oxidized in a graded way by increasing irradiance with light.

MATERIALS AND METHODS

Isolation of retinal epithelial cells. Experiments were performed in isolated RPE cells taken from bovine eyes freshly obtained from a local abattoir. The anterior seg-

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*To whom correspondence should be addressed.
†*Abbreviations*: AA, ascorbic acid; DHA, dehydro-L-ascorbic acid; DOPA, dihidroxyphenylalanine; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HPCL, high pressure liquid chromatography; RPE, retinal pigmented epithelium.

Figure 1. Electron micrograph of isolated retinal pigment epithelial cell. Suspensions of RPE cells were prepared from fresh bovine eyes as described in the text. A sample of the cells was immediately fixed in buffered glutaraldehyde fixative and prepared for EM analysis. Cells appear to be intact, including their pigment granules. Magnification 2880×.

ment was dissected from the eyes, and the retina was removed by grasping it with blunt forceps and lifting it so that it could be cut free at the optic nerve. The eye cup was rinsed three times in 0.32 *M* sucrose. One mL of 0.32 *M* sucrose was placed in the eyecup and RPE cells were carefully brushed from the choroid with a soft camel hair brush. Cells from up to 30 eyes were combined for use in the experiments. The cells were concentrated by centrifugation for 10 min at 1000 *g*. Examination by light and electron microscopy of the cell suspensions revealed that the cells remained intact after this treatment (Fig. 1). Typically, 10 bovine eyes produced cell suspensions containing 1 to 6 × 10⁷ RPE cells/mL and 6 to 8 × 10⁶ erythrocytes/mL.

Reaction mixture for determination of ascorbic acid oxi-

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dation. The reaction of RPE cells, AA, and light was carried out in a total volume of 50 μL. This was made up of a 25 μL aliquot of RPE cells with the balance of the volume comprised of 0.2 mM labeled AA (New England Nuclear, Boston, MA, specific activity 6893 cpm/nmol), 0.32 M sucrose, and 0.05 mM EDTA. The EDTA was added to minimize oxidation by trace amounts of divalent metal ions, although we note that copper chelates may still be capable of catalyzing some oxidation of AA (Buettner, 1986). The effect of DHA was tested at concentrations of 1 and 5 mM.

Ascorbic acid assay. The DHA was separated from AA by HPLC on a μ-Bondapak–NH$_2$ column (Waters Associates, Milford, MA), eluted with 20 mM NH$_4$H$_2$PO$_4$ at a rate of 1 mL/min (Fox *et al.*, 1982). The retention times of AA and DHA were determined by the elution from the column of [^{14}C]AA before and after oxidation by CuSO$_4$. The eluant was passed through a UV detector (Waters Associates Model 441 with a 280 nm filter), mixed with scintillation fluid (Radiomatic, Flow Scint III), and assayed for radioactivity with an in-line detector (Radiomatic, Meriden, CT, Flo-One Beta A-250). Flow rate of scintillation fluid through the beta detector was 4 mL/min, and radioactivity was recorded every 6 s.

Oxidation of AA was assayed in the reaction mixture immediately following exposure of the mixture to a visible laser. The sample tubes containing the exposed reaction mixture were centrifuged at 800 g for 1 min to precipitate the cells. Ten mL of supernatant were removed and injected onto the μ-Bondapak–NH$_2$ column.

Laser exposures. Laser light was derived from a Coherent (Palo Alto, CA) Model 920 argon-krypton laser photocoagulator. The output of this laser system was selected to be either the mixed blue–green emission of the argon laser (approximately 55% 488.1 nm and 45% 514.5 nm), or the krypton red emission (647.1 nm). A fiber optic conducted the laser output into a 10× expander which produced a final beam diameter of approximately 20 mm. In order to expose the cells to the laser, 25 μL of the RPE cell suspension were placed with 25 μL of reaction mixture in a plastic micro-centrifuge tube clamped so that the laser beam entered the tube at its mouth. Because the beam diameter was larger than that of the sample tube, the cells were completely and uniformly covered (ignoring self-screening of the interior of the sample) by the laser beam. The power output of the laser was controlled by adjusting the laser tube current, and the exposure duration was limited by means of a shutter at the laser exit aperture. A Laser Precision (Utica, NY) DG-10 thermopile radiometer was used to measure the beam power at the sample tube, and the sample irradiance was calculated in mW/cm^2.

Cell fractionation and preparation of melanin granules. The RPE cells were suspended in 0.32 M sucrose, centrifuged at 800 g for 10 min, and resuspended in 0.32 M sucrose to a volume of 1.25 mL. One mL was subjected to sonication for 15 s. No intact cells remained after sonication, but the number of free pigment granules was increased. The sonicated cells were then subjected to successive centrifugation at 800 g for 10 min, 8000 g for 10 min, and 100000 g for 2 h. Pellets were obtained after each centrifugation, resuspended in 1 mL of 0.32 M sucrose, and identified as "P1", "P2" and "P3" fractions, respectively. A 20 μL aliquot of each fraction was combined with 0.05 mM EDTA and 0.2 mM [^{14}C]AA to a final volume of 50 μL. The mixture was then exposed to the laser and assayed for DHA production in the usual manner.

RESULTS

Separation of ascorbic acid and dehydro-L-ascorbic acid

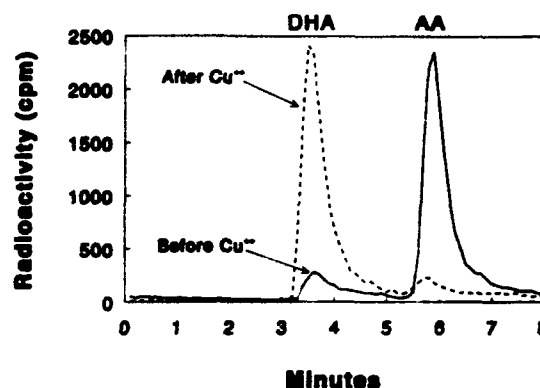The retention times of AA and DHA were determined by running a known standard of each on

Figure 2. Separation of AA and DHA by HPLC. For analysis of AA oxidation, samples were placed on a μ-Bondapak–NH$_2$ column and eluted with 20 mM NH$_4$H$_2$PO$_4$ at a flow rate of 1 mL/min. To establish the retention time of DHA, 0.2 mM [^{14}C]AA in 10 mM ammonium phosphate was incubated with 0.4 mM CuSO$_4$, which resulted in nearly complete oxidation of AA to DHA. The figure shows the elution of radioactivity before and after incubation with CuSO$_4$.

the μ-Bondapak–NH$_2$ column. When [^{14}C]AA was incubated in 0.4 mM CuSO$_4$, the AA was oxidized within minutes to DHA. On the μ-Bondapak–NH$_2$ column, DHA eluted first (retention time 3.5 min), while AA eluted at about 6 min (Fig. 2). Because DHA has negligible absorption at 280 nm, only the ascorbate peak is revealed by the UV detector. The elution of radioactivity in the DHA peak, however, indicated that oxidation of the [^{14}C]AA occurred. Complete separation of AA and DHA was achieved under these HPLC conditions.

Effect of laser exposure conditions

The test solution containing [^{14}C]AA was added to RPE cell suspensions and the mixture was exposed to the laser output for various durations. The irradiance of the sample by the laser was held constant during the exposure period and only the duration was varied. Under these conditions, the oxidation of AA to DHA was demonstrated, but the extent of AA oxidation was dependent on the experimental conditions, summarized in Table 1. When RPE cells were present in the test mixture but not exposed to the laser, a low level of AA oxidation was observed, possibly due to the effects of ambient room light. In fact, slightly more labeled DHA was produced in a sample of RPE cells stored for 5 h in ambient room light than in a similar sample stored for 5 h in the dark. The extent of endogenous oxidizing activity of the RPE cells has not yet, however, been thoroughly investigated. When RPE cells were omitted from the test mixture, there was negligible oxidation of AA by the laser, even when cold DHA was present. Thus the oxidizing action of the laser depended on some factor present in the RPE cells.

When the test solution containing RPE cells and (nominally) 14.3 nmol of AA was exposed for 60 s

Table 1. Effect of DHA and ambient light on AA oxidation by RPE cells

Experimental conditions					Results	
Cold DHA (mM)	RPE	Ambient light	Laser exp. time (s)	N	DHA (nmol)	AA (nmol)
5	−	0*	60	4	0.10 ± .17	14.88 ± .82
5	+	0†	0	2	0.57 ± .02	11.73 ± .06
5	+	5h‡	0	2	0.85 ± .01	11.80 ± .25
0	+	0*	60	2	1.71 ± .01	12.78 ± .61
5	+	0*	60	2	4.31 ± .23	9.60 ± .14

Where $N = 2$, results are expressed as the average ± high and low values, otherwise the results are the average ±1 SD.
*Includes 5 min processing time under ambient light.
†Stored 5 h in dark.
‡Stored 5 h in ambient laboratory light.

to the blue–green argon laser output at an irradiance of 90 ± 2 mW/cm², 1.71 nmol of AA (about 12% of the available AA) was oxidized to DHA. We hypothesized that if the RPE is capable of reducing DHA to AA, more AA may be oxidized by the laser exposure than was apparent in the first experiment. To test this possibility, we added unlabeled DHA to the reaction mixture prior to the laser exposure. If oxidation–reduction cycling is occurring, "cold" DHA should compete with the ^{14}C-labeled pool of DHA for conversion back to AA, thereby trapping additional label in the DHA fraction. Consistent with this prediction, when unlabeled DHA was present in the reaction mixture, 4.3 nmol of AA was oxidized by the laser exposure (Table 1), which represented about 30% of the available AA.

Effect of laser exposure time

An approximately linear relation was observed between radiant exposure and DHA production for exposures of 15–120 s, with the sample irradiance held constant at 90 ± 2 mW/cm² (Fig. 3). A threshold for the oxidative effect was not observed within the range of exposure duration used in these experiments. A linear regression line fit to the data points did not have a zero Y-intercept, probably due to some endogenous oxidizing activity in the RPE cells. In comparison, a cell-free control had nearly zero AA-oxidizing activity (Fig. 3). Also shown in Fig. 3 is the amount of [14C]AA remaining in the sample following laser exposure. The total radioactivity recovered in the AA and DHA fractions from the chromatograms remained approximately constant; AA is evidently not being converted to any species other than DHA.

Effect of laser wavelength

The output of the laser was tuned to the krypton red wavelength (647.1 nm) and the exposure power was adjusted to be radiometrically equal to the

Laser: Argon Blue-Green   Cold DHA Present
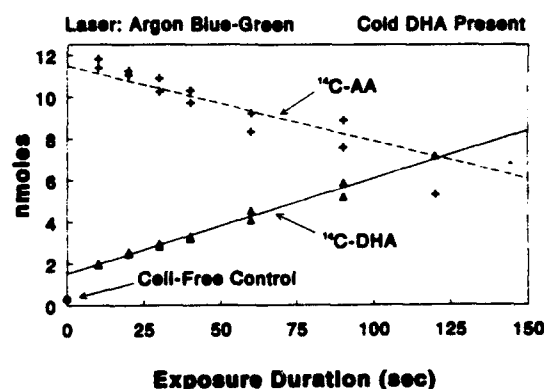
Exposure Duration (sec)

Figure 3. Effect of exposure duration on AA oxidation. Samples of the AA reaction mixture with 5 mM DHA were exposed to the argon laser for various durations at an irradiance of 90 ± 2 mW/cm². The amount of AA oxidized was a linear function of the exposure duration ($r = 0.969$), at least over the range of 10 to 120 s. Some endogenous oxidizing activity is apparently present in the RPE cells, as indicated by the non-zero Y-intercept of the regression line fit to the DHA data points. There is negligible oxidation in cell-free, exposed or unexposed samples.

blue–green output. The amount of DHA produced by the red light exposures was much less than that produced by the shorter wavelength light (Fig. 4), despite the smaller number of photons delivered by the blue–green light. On a quantal basis, therefore, red light is less effective than the blue–green light in driving the conversion of AA to DHA.

Localization of the photosensitizer

The RPE cells were subjected to a series of manipulations in order to identify the cellular component responsible for the light-mediated AA oxidation. The capability to oxidize AA during light exposure was not lost following sonication, nor by heating the cells to 80°C. Prior to sonication, the cell suspensions contained 5.4×10^7 RPE cells/mL, 6.2×10^6 red blood cells/mL, and 1×10^9 pigment granules/mL. After sonication, no intact cells were
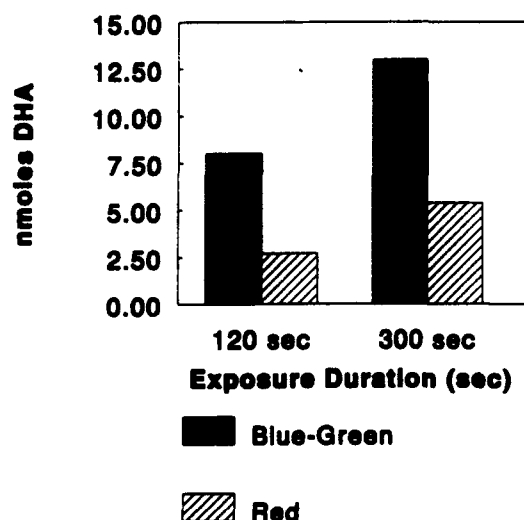
Figure 4. Effect of laser wavelength. Samples of the AA reaction mixture plus 1 mM DHA were exposed to either the argon blue–green (488.0 + 514.5 nm) or the krypton red (647.1 nm) output at 90 ± 2 mW/cm² for 120 or 300 s. The amount of DHA produced by the laser exposure was determined by HPLC. The shorter wavelength light was at least twice as effective as the long wavelength in initiating AA oxidation.
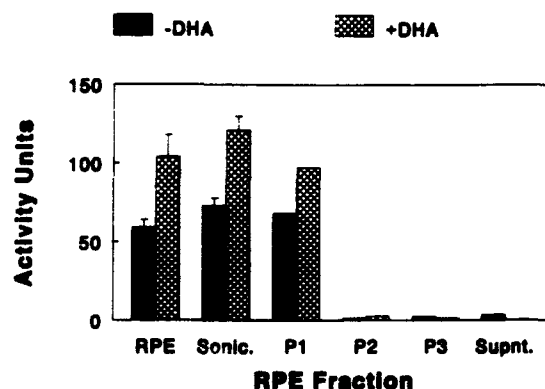
Figure 5. Localization of AA oxidizing activity. The ability of whole RPE cells to promote AA-oxidation was compared to sonicated cell fragments and to various subcellular fractions. Legend: RPE = whole cell sample (approx 10⁸ cells); Sonic = sonicated cells; P1 = pellet from centrifugation at 800 g for 10 min (approx 2 × 10⁹ free pigment granules); P2 = pellet from centrifugation at 8000 g for 10 min; P3 = pellet from centrifugation at 100000 g for 2 h; Supnt. = supernatant resulting from final, high-speed centrifugation. Each of these fractions was added to the AA reaction mixture, with and without unlabeled DHA, and exposed to the argon blue–green laser for 60 s. The graph shows the units of activity of each sample, where 1 unit = 1 nmol of DHA produced in 50 μL of sample.

present, but the granule concentration increased to 1.9 × 10⁹/mL, apparently because they were released from disrupted cells. The sonicated cells were fractionated as described in the Methods, exposed to the argon laser for 60 s, and the oxidizing activity of each fraction was measured and expressed in units of activity (1 unit = 1 nmol of DHA produced per 50 μL reaction medium). As indicated in Fig. 5, nearly all of the AA oxidizing activity was retained in the P1 fraction. Examination of the fractions by light microscopy revealed that most of the pigment granules were precipitated during the low-speed centrifugation, and that the granules were the most prominent feature of P1. Thus, the photosensitizing activity appeared to be associated with the melanin granules.

DISCUSSION

Our findings indicate that a fast, photooxidative process occurs in the environment of the RPE during light exposure. The products of this process, probably free radicals, are capable of reacting quickly with ascorbic acid, which thereby presumably reduces free radical toxicity. We note that the oxidation produced by a given light exposure is essentially complete at the end of the exposure. Although hydrogen peroxide has often been proposed as an intermediate in ocular light damage, we have found the direct oxidation of AA by hydrogen peroxide-generated OH⁻ proceeds much more slowly than the light-mediated reaction (unpublished observation). Therefore, another free radical species may be responsible for AA oxi-

dation. The observation that unlabeled DHA added to the reaction mixture increases the radioactivity in the DHA fraction provides evidence that AA is able to undergo oxidation–reduction cycling in the RPE. Such a process, by maintaining the availability of reduced AA, enhances the ability of the antioxidant to protect ocular tissues from light damage by scavenging free radicals.

The oxidation of AA following light exposure is especially interesting in view of the antioxidant and free-radical scavenging role AA is supposed to play in ameliorating ocular light damage (Organisciak et al., 1985; 1987; Li et al., 1985; Noell et al., 1987). Moreover, after induction of light damage, the amount of AA is reduced in the RPE and choroid (Woodford et al., 1983; Tso et al., 1984). These previous studies however, observed changes in AA oxidation after chronic or damaging light exposures. The present study observed alterations in AA oxidation after relatively brief exposures below the intensity required for visible retinal damage (ANSI, 1989). The linear dependence of DHA production upon total light exposure suggests an application of this reaction in the quantification of ocular photooxidative stress. For example, two electrons must be transferred from AA to an acceptor to complete the oxidation of AA through its semidehydroascorbic acid intermediate to DHA. In the absence of competing reactions, the stoichiometry of this reaction may be used to estimate the number of free radicals produced by a given light exposure.

The wavelength sensitivity of the light-triggered AA-oxidation suggests that a specific sensitizer is

involved in the reaction. Our experiments provide direct evidence that a sensitizer resides in the RPE, which absorbs at least 50% of the visible light entering the primate eye, depending on the wavelength of the light and the age of the eye (Boettner and Wolter, 1962). Other evidence that the RPE contains a sensitizer for light-initiated reactions exists. The earliest photochemical lesions involve the RPE and photoreceptors (Ham et al., 1986). Although a specific sensitizer has not yet been identified, several studies have indicated that short wavelength light is especially damaging (Ham et al., 1979; Pautler et al., 1989; Dorey et al., 1990). Pautler (1990) suggested that a heme-containing protein, probably a cytochrome, was the sensitizer for some light-initiated changes in RPE cell function, although aromatic amino acids or other intermediates involved in melanogenesis are also candidates (Dorey et al., 1990). Our results are consistent with these previous studies in that short-wavelength, visible light is particularly effective in triggering an oxidative process. However, we have demonstrated that the photooxidation of AA is unaffected by sonication of the RPE cells, or by heating cell fractions to 80°C. These observations indicate that a non-enzyme sensitizer, such as melanin, is the sensitizer involved in the oxidation of AA. Furthermore, the majority of the oxidizing activity is associated with a subcellular fraction rich in melanin pigments.

Although melanin pigments have often been proposed as serving a protective role against photooxidative stress (e.g. Menon and Haberman, 1979; Sakina et al., 1985; Ostrovsky et al., 1987), the melanin heteropolymer can generate free radicals when irradiated with visible light (Mason et al., 1960; Cope et al., 1963). Melanin's free radical nature has been proposed to result from its existence as a mixture of quinone–hydroquinone and quinone–semiquinone polymers (Longuet–Higgins, 1960). Recent work has supported this proposal by examining the fluorescent spectrum of DOPA–melanin (Gallas and Eisner, 1987). The melanin radical is relatively stable. The EPR peak height in bovine eye melanin samples irradiated with white light from a tungsten lamp required 2–3 s to return to baseline after the light was extinguished (Cope et al., 1963). Thus, sufficient irradiation of melanin could produce excess free radicals capable of reacting with surrounding tissues or molecules. It must be noted, however, that reactivity of melanin radicals is determined by additional factors such as the type of melanin (Sealy et al., 1984), and whether the melanin is complexed with proteins (Menon et al., 1976). Moreover, the ability of melanin to participate in rapid electron transfers is suggested by the presence of a bipolar peak in photocurrent which reaches a maximum with irradiation by 500 nm light (Crippa et al., 1978). The argon laser emissions used in the present work bracket this wavelength. Further work will be necessary to eluci-

date the interactions between the ascorbic acid redox cycle and the production of free radicals in the ocular melanins during light exposure in vivo. In the interim, the oxidation of AA by reactive photoproducts appears to be a useful method for studying the effects of low-power lasers and other light sources on the ocular tissues.

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Prevention of light-induced free radical production from melanin granules by ascorbic acid.

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INTRODUCTION

Increased ascorbic acid oxidation in the retinal pigment epithelium (RPE) after prolonged exposure to light [1-3] led us to postulate a possible role of ascorbic acid in the prevention of light damage to the retina. However, the mechanism by which ascorbic acid functions as an antioxidant is uncertain. Ascorbic acid may serve as a substrate for peroxidase to prevent intracellular accumulation of hydrogen peroxide [4]. On the other hand, ascorbic acid may be able to react directly with light-induced free radicals. The majority of light energy entering the eye is absorbed in the retinal pigment epithelium [5], therefore, it is likely that an efficient means of detoxifying light-induced free radicals exists in the RPE. We have previously demonstrated rapid ascorbic acid oxidation during exposure of the RPE cells to light [6]. Because this rapid reaction is a suitable candidate for a detoxification mechanism, and because it appears to be dependent on the presence of melanin, we designed the present study to characterize the light-dependent ascorbic acid oxidation reaction in the presence of melanin granules in a cell free incubation mixture.

METHODS

Preparation of retinal pigment epithelial cells. Intact RPE cells were prepared from freshly obtained bovine eyes by the method previously described [6]. Melanin granules were prepared from cells suspended in 1 ml sucrose and disrupted by a sonicator. The suspension was layered on 40 ml of 2 M sucrose and subjected to centrifugation at 8000 g for 30 minutes. The pigment granules were washed once more in the same manner, then suspended in 0.25 M sucrose with 0.05 mM ethylenediaminetetraacetic acid (EDTA). The concentration of pigment granules was estimated from counts made with a hemocytometer.

Light induced ascorbic acid oxidation. [^{14}C]-ascorbic acid (20 nmol, specific activity 4500 cpm/nmol) purchased from New England Nuclear (Boston, MA) was mixed with melanin granules in a final volume of 0.05 ml containing 0.05 mM EDTA. The suspension was illuminated with an Argon-ion, continuous-wave laser. The mixed, 488.1 and 514.5 nm output was used, at irradiances and for durations as indicated in the text. At the end of the laser exposure, the suspension was passed through a 10M millipore filter and 0.02 ml of the filtrate was injected into a μ-Bondapax-NH$_2$ column and eluted by 10 Mm ammonium phosphate. Under these conditions, the retention time of dehydroascorbic acid (DHA), the oxidized form of ascorbic acid, was 3.5 min, and that of ascorbic acid was about 6 min. The eluant was passed though a Waters Associates model 190 UV detector (280nm filter) and then a Radiomatic model A200 radioactivity detector.

Glutathione oxidation. 12.5 μl glutathione (0.1 M) was mixed with 12.5 μl of melanin (10^9/ml) and exposed to the laser for 2 min. Reduced and oxidized glutathione were determined by high

pressure liquid chromatography using a Waters Associates Delta column, eluted by a mixture of 3% acetonitrile and 0.1% trifluoroacetic acid. Absorbance was monitored at 210 nm.

RESULTS

In our recent report [6], we described light-dependent ascorbic acid oxidation mediated by RPE cells. The reaction, however, does not require intact cells, and is also enhanced by the presence of DHA in the reaction mixture, as was the case for intact cells (Table 1). Most of the activity was recovered in the fraction containing the melanin granules, following sonic disruption of the cells. For a given exposure, the amount of DHA produced by the laser exposure was proportional to the log of the melanin granule concentration over the range studied (Figure 1).

Table 1
Effect of sonication and DHA on Photooxidation of Ascorbic Acid.
Effect of Sonication

	nmol DHA formed after 2 min laser	
	+10^6 RPE cells	+10^6 granules[2]
Before sonication	19.22 ± 2.6 (3)[1]	1.04 ± 0.14 (3)
After Sonication	22.96 ± 1.58 (3)	0.62 ± 0.04 (3)

Effect of DHA		-DHA	+DHA[3]
	Initial	After Laser Exposure[4]	
[^{14}C]DHA produced	0	0.86 ± 0.13 (4)	1.22 ± 0.18 (7)
[^{14}C]AA remaining	7.69 ±0.35	5.87 ± 0.55 (4)	6.05 ± 0.42 (7)

[1]number of analyses specified in parenthesis
[2]Granules purified by centrifugation on 2 M sucrose. 5.16 x 10^6 granules/assay. Incubation volume was 50 µl.
[3]DHA concentration: 5 mM.
[4]Laser Exposure: 90 mW/cm^2 for 120 sec.

In our previous investigations, the oxidation of ascorbic acid commenced with the onset of the light exposure. To investigate this further, we examined the time course of the reaction during and after the light exposure. The experiment shown in Figure 2 illustrates that the reaction occurs only during light exposure, and ceases as soon as the light is turned off. Consecutive analyses of the reaction mixture following the laser exposure reveal that the ratio of oxidized and reduced ascorbic acid remains stable, i.e. once the light is extinguished, there is no further ascorbic acid oxidation. Because ascorbic acid oxidation begins as soon as the light is turned on and stops immediately after the light is turned off, it is likely that ascorbic acid reacts directly with the light-activated melanin.
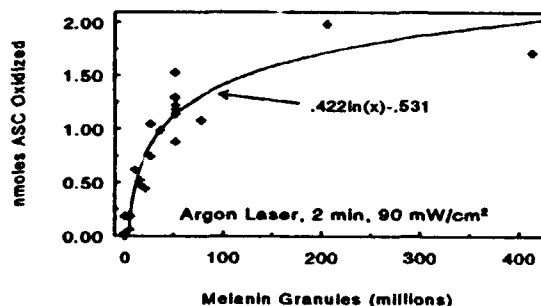
Figure. 1. The rate of ascorbic acid oxidation is dependent on melanin concentration. Retinal pigment epithelial cells brushed from 10 bovine eyes were suspended in 40 ml of 0.25 M sucrose and recovered by centrifugation at 2000 rpm for 10 min. The cells were resuspended into 1 ml sucrose. The cell suspension contained 5.4×10^7 RPE cells/ml, 6.2×10^6 red blood cells /ml, and 1.0×10^9 free pigment granules/ml.
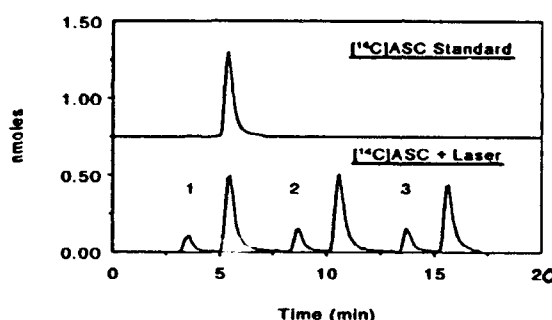

Figure 2. Termination of ascorbic acid oxidation after the laser exposure. Top: Ascorbic acid (ASC) standard. Bottom: 20 nmol ^{14}C-ascorbic acid incubated with 10^6 melanin granules and exposed to the laser for 2 min at 90 mW/cm^2. The suspension was filtered through a millipore membrane, and 10 µl of the filtrate was injected three times into a µ-Bondapax-NH$_2$ column. The large peaks are ASC; the three consecutive DHA peaks are labeled 1, 2, and 3.

Because GSH is present in ocular tissues and participates in an oxidation-reduction cycle through its oxidized intermediate (GSSG), we examined the extent to which glutathione could be oxidized by light-activated melanin. Glutathione and oxidized glutathione were analyzed by high pressure liquid chromatography capable of detecting picomole amounts of oxidized glutathione. There was no significant oxidation of glutathione after exposure to light (Figure 3).

DISCUSSION

The present observations document the occurrence of an efficient conversion of light energy into chemical energy in the retinal pigment epithelial cells. This reaction is very specific to ascorbic acid; for example, the other cytoplasmic reducing agent, glutathione, is ineffective. The rapid ascorbic acid oxidation
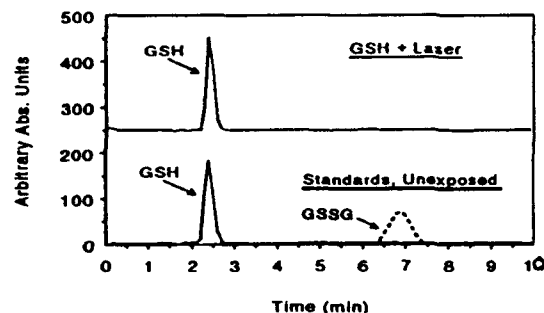
Figure 3. Glutathione does not react with light-activated melanin granules. Top: Reduced glutathione (GSH) and melanin granule mixture was exposed to laser for 2 min at irradiance of ~400 mW/cm². 2 µl aliquot then injected into Delta HPLC column. Bottom: GSH (200 nmol) and GSSG (32.6 nmol) standards separated on Delta column.

converts light energy at moderate fluences to chemical energy instead of heating the melanin granules. As shown by studies of thermal damage mechanisms, hot melanin granules are toxic to the RPE cells and photoreceptors [7].

Ascorbic acid and glutathione are the two major reducing agents in the cytoplasm. Although cytoplasmic glutathione may play a role in regenerating ascorbic acid from dehydroascorbic acid [8], glutathione apparently does not react with the energized melanin granules. Therefore, the present data indicate that ascorbic acid plays a very specific role in the photooxidative reaction of melanin and is probably not replaceable by other physiological reducing agents. These findings indicate that ascorbic acid oxidation, coupled to light-activated melanin granules, converts excess light energy to chemical energy and thus prevents activated melanin granules in the form of heat or as reactive radical species.

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SODIUM DEPENDENT ASCORBIC AND DEHYDROASCORBIC ACID

UPTAKE BY SV-40 TRANSFORMED RETINAL PIGMENT EPITHELIAL CELLS

by

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Running title: AA and DHA uptake

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SUMMARY

The present data confirmed previous studies with other cell types that ascorbic acid and dehydroascorbic acid are transported through different transporters into SV-40-transformed retinal pigment epithelial cells. These experiments were performed on cells grown on 96-well culture plates. Ascorbic acid was taken up into the cell by a high affinity transporter with K_m=0.041 mmol/L and a low V_{max} of 2.74 pmol/min/well. Dehydroascorbic acid was taken up by a low affinity transporter, with K_m=5.67 mmol/L, however, the V_{max} was 325.5 pmoles/min/well. Both ascorbic acid and dehydroascorbic acid uptake were dependent on sodium concentration. The uptake of ascorbic acid does not involve oxidation-reaction steps because the uptake of [^{14}C]-ascorbate was unaffected by the presence of an excess amount of unlabelled dehydroascorbic acid.

INTRODUCTION

The ability of eye tissues to extract ascorbic acid (AA) from the blood circulation is well known. Friedenwald et al(1) postulated that the active transport of AA across the ciliary epithelium is the driving force of aqueous humor formation. Their hypothesis was strengthened by recent observations on sodium dependent AA transport into the ciliary body epithelium (2,3). The high concentration of AA in the posterior segment of the eye may have a role as an antioxidant to prevent photooxidative damage to the retina. The involvement of AA in detoxification of light induced free radicals was supported by the observations of light-dependent AA oxidation in the retinal pigment epithelial cells (4); increased dehydroascorbic acid (DHA) in the retinal pigment epithelium of guinea pigs (5) and primates (6) after prolonged exposure to light; and the ability of AA to protect the retina from light damage in rats (7). It is therefore important to understand how AA is mobilized across the pigment epithelium so that the AA concentration in ocular tissues can be artificially regulated for therapeutic purposes.

AA transport has been studied in erythrocytes (8) leukocytes (9,10,11), intestine (12,13), brain (14), cat retinal pigment epithelial cells (15), and ciliary epithelium (2,3,16). Tissue cells have a sodium-dependent, high affinity transporter for AA. Therefore, at the normal physiological concentrations of AA and Na^+ in the blood, AA is actively transported into tissue cells at the maximal rate supported by the active transport apparatus (17,18). The detailed mechanism by which AA is transported across plasma membrane is uncertain.

Because DHA is more lipid soluble than AA, earlier investigators (1,9) speculated that AA was oxidized prior to uptake into the cells. Recent investigations of leukocytes (11), and ciliary epithelium (16), indicated that AA and DHA are transported into the cell through different mechanisms. However, the currently available data do not rule out the possibility that AA oxidation-reduction occurs at the site of AA transport. The present study was directed to compare the kinetic properties of AA and DHA uptake into SV-40 transformed human retinal pigment epithelial cells to determine if AA is oxidized during transport into the cells.

METHODS

SV-40 transformed human RPE Cells: This cell line was obtained from Dr. Corinne G. Wong, Department of Ophthalmology, the University of California at Irvine. This particular subculture of RPE cells has been characterized and compared with normal RPE cells (19,20). The cell line was originally obtained from the permanent line RPE 28 SV4 in the NIH Aging Cell Repository. It was derived by transfecting human fetal RPE cells with SV-40 virus, strain RH 911. For long-term storage, the cells were kept in liquid nitrogen. Frozen cells were thawed and cultured in 75 cm^2 culture flasks. Confluent RPE cells were passaged every 2 weeks, and the medium was renewed twice a week. Cells were routinely maintained in the culture system for at least one month before experimentation. For experimentation, 96-well culture plates (Corning) were plated with RPE cells at a seeding density of 3.2 X 10^4 cells/ml. After a week, the cells reached confluence at a density of 7300 cells/mm^2 so that there were

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about 2.3 X 10⁵ cells/well. The average protein content in 16 wells determined by the method of Lowry et al (21) was 0.15 ±0.01 mg/well.

Incubation medium: The complete culture medium used was Ham F12 (Cellgro) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin-streptomycin (Gibco), and fungizone (Gibco). The complete culture medium was used in all experiments except those involving the effect of NaCl.

The medium described by Helbig et al (16) was used to study the effect of sodium on AA uptake. The control incubation medium was a Ringer solution containing 151 mmol/L NaCl, 4 mmol/L KCl, 1.7 mmol/L $CaCl_2$, 1 mmol/L KH_2PO_4, 5 mmol/L D-glucose and 0.9 mmol/L $MgSO_4$ buffered by HEPES at pH 7.4. In solutions containing low sodium concentration, NaCl was replaced by N-methyl-D-glucamin (NMDG). In the sodium free medium, NaCl was completely replaced by 151 mM NMDG.

Measurement of AA uptake: [¹⁴C]-AA was purchased from New England Nuclear. The specific activity was 12,000 cpm/nmol. The cells were grown in 96-well plates. At the beginning of the experiment, the culture medium was removed rapidly by suction, replaced by 0.1 ml of fresh culture medium containing a specified amount of [¹⁴C]-AA and unlabeled DHA, and incubated at 37 °C for 30 minutes. At the end of incubation, this culture medium was removed by suction, and the wells were rinsed 4 times with 0.25 ml of culture medium.

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In order to determine total cellular [^{14}C]-AA uptake, the cells in each well were extracted by 0.25 ml of 0.2 N NaOH, and 0.2 ml of the extract was mixed with 5 ml of scintillation fluid. Radioactivity was determined in a LKB Model 1209 RACKBETA counter.

The cells were extracted in the culture wells by 0.02 ml methanol as described by Washko et al (11) in order to determine the intracellular proportion of AA and DHA. The extract from 4 wells was combined and 0.025 ml of the methanol extract was injected into a μ-Bondapax-NH$_2$ column (Waters Associate,Medford,MA). The column was eluted by 20 mmol/L ammonium phosphate. The eluant was allowed to pass through a UV detector with a 280nm filter, then mixed with scintillation fluid (4ml/min) and passed through the 2.5 ml detector cell in a Radiomatic model A250 radioactivity flow detector. The retention time of DHA and AA was 3.5 and 7.6 min respectively.

The uptake of unlabeled DHA was estimated from the increased AA in the cells. The cells were incubated with a specified concentration of DHA for 30 min. At the end of incubation, the incubation medium was removed, and the remaining cells were washed 4 times with 0.25 ml of culture medium. The cells were extracted by 0.1 ml methanol, and 0.01 ml of the methanol extract was analyzed for AA by the high pressure liquid chromatography method described above. Washko et al(11) has shown complete extraction of AA from granulocytes by methanol. Because methanol has negligible effect on our chromatographic method for analysis of AA, methanol was chosen to extract AA from the cells.

Kinetic data was analyzed by the use of the computer program described by Bliss and James (22).

RESULTS

When [^{14}C]-AA was incubated with the cells in the complete culture medium, the uptake of radioactivity into the cells was linear for at least 30 min (Figure 1). Therefore, 30 min incubation was used in all kinetic studies.

[^{14}C]-AA uptake exhibited first order saturation kinetics as shown in Figure 2. The rate of uptake is dependent on AA concentration in the culture medium, and reached a maximal rate of 2.74 pmol/min per well, with a K_m value of 0.041 mmol/L (Table 1).

The influence of NaCl concentration on AA uptake is illustrated in Figure 3. In the low sodium medium, [^{14}C]-ascorbate was taken up into the cells at a maximal rate of 0.4 pmol/min/well. A sharp rise of AA uptake was observed as NaCl concentration increased above 0.1 mol/L and reached a maximal rate of about 1.55 pmol/min/well.

When only DHA was incubated with the cells, AA inside the cells increased. There was negligible DHA inside the cells. The increased intracellular AA was used to infer the rate of DHA uptake, assuming that all DHA entering the cells was converted to AA. The increased AA concentration in the cells was also dependent on extracellular DHA

concentration, exihibiting first order kinetics (see Figure 4). However, the K_m (5.67 mmol/L) and V_{max} (325.5 pmol/min/well) of DHA uptake were much higher than that of AA (Table 1).

The addition of non-radioactive DHA to the wells did not inhibit uptake of [^{14}C]-AA (Table 3). The non-competitive relationship of AA and DHA is qualitatively illustrated in Figure 5. After incubation of [^{14}C]-AA with the cells, radioactivity was observed only in the AA peak (Figure 5, trace "B"). The retention time of DHA was 3.8 min. as previously established (4). There was negligible radioactivity in the expected location of DHA. If [^{14}C]-AA were oxidized and reduced as it entered each cell, then unlabelled DHA would have competed with the reduction of [^{14}C]-DHA resulting in residual [^{14}C]-DHA in the cell extract. The present data showed negligible [^{14}C]-DHA in the cell extract in the presence of an excess amount of unlabelled DHA (Figure 5, trace "A"). DHA uptake was also dependent on sodium concentration. After disrupting the cell by sonication, DHA was not reduced by the cell homogenate. Therefore, reduction of DHA was dependent on the active metabolism of the living cell.

DISCUSSION

The present data show that AA is taken up into the transformed cells by a high affinity transporter and the rate of uptake is dependent on sodium concentration as observed by others using normal tissues or blood cells (11-16,23,24). The K_m observed for the transformed cells is very similar to that reported for normal leukocytes (11) and ciliary

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epithelial cells (15). Therefore, it is likely that the mechanism of AA uptake into the SV-40 transformed RPE cells is very similar to that of normal cells.

One of the objectives of this study was to determine if AA is oxidized during transport into cells. Because DHA is more hydrophobic than AA, previous investigators (8,9) speculated that AA is oxidized prior to transport. Their speculation was based on their observation that DHA was taken up faster than was AA. The recent studies with leukocytes (11), embryonic brain tissues (14) and cultured ciliary epithelium (16) indicated that AA and DHA were taken up through different channels. However, Washko et al (11) pointed out that their data did not exclude the oxidation of AA bound to the cell membrane followed by immediate reduction as it entered the cytoplasm. The absence of competition between unlabelled DHA and [^{14}C]-AA during uptake, observed in the present study, led us to conclude that the high affinity transporter for AA does not involve oxidation-reduction to move AA across the cell membrane. If prior oxidation were required for AA transport into the cells, the uptake of ^{14}C-AA would have been inhibited by the presence of an excess amount of unlabelled DHA.

Furthermore, the hypothesis that AA oxidation occurs at the time of crossing the cell membrane includes a speculation that oxidized AA is reduced after it enters the cell. If this premise were correct, the unlabelled DHA entering the cells would have competed with the hypothetical reduction of [^{14}C]-DHA produced in the cell membrane, resulting in the accumulation of [^{14}C]-DHA in the cell. On the contrary, only [^{14}C]-AA was seen in the cells'

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extract when an excess amount of DHA was added to the culture medium containing a small amount of [^{14}C]-AA (experiment illustrated in Figure 5).

Khatami et al (25) observed inhibition of [^{14}C]-AA transport into primary culture of cat retinal pigment epithelial cells by unlabelled dehydroascorbate. Their cell preparations contained high amounts of pigment granules. We have previously demonstrated oxidation of AA by the melanin granules (4). It is likely that in the study of Khatami et al.(25) the [^{14}C]-AA added to the pigmented cell culture was partially oxidized to [^{14}C]-DHA, and the radioactivity taken up into the primary culture of pigmented cells included both [14C]-AA and [^{14}C]-DHA. The addition of unlabelled DHA, therefore, would have appeared to inhibit partially the uptake of labelled AA into the pigmented cells. The transformed retinal pigment epithelial cells used in the present study did not contain pigment granules and therefore the oxidation of AA was negligible.

A comparison of AA and DHA uptake led us to observe a marked difference between their rate of uptake into the cells. AA is taken up into the cells by a high affinity transporter (Km=0.041 mmol/L) while DHA is transported into the cells by a low affinity transporter (Km=5.67 mmol/L) and both are dependent on extracellular sodium.

Helbig et al (16) observed a negligible effect of Na$^+$ on DHA uptake into ciliary epithelial cells. The effect of Na$^+$, however, was studied only at 0.025 mmol/L DHA and AA. In the present study, the complete kinetic data for DHA over the concentration of 0-10

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mmol/L was obtained. In the presence of 0.025 mmol/L DHA, the rate of DHA uptake is far below the optimal rate required to visualize the sodium effect. At the optimal concentration of DHA, the stimulatory effect of sodium was obvious as shown in Table 3.

The AA concentration in blood is 0.01-0.06 mmol/L which is near the K_m for AA uptake by tissue cells. Therefore, the retinal pigmented epithelium should be capable of extracting AA from the choroidal blood near the maximal rate. DHA in the blood is only a very small fraction of total AA, and the rate of uptake under the normal physiological condition is negligible. However, AA uptake reaches a maximal rate of 2.7 pmole/min, while that of DHA can approach a V_{max} of 325 pmole/min/well (Table 1) in the presence of Na^+ at physiological concentration. In recent years, AA has been shown to have a role as a physiological antioxidant (4,7). Because the rate of AA uptake is already near maximum at normal physiological blood AA concentration, it is difficult to increase, through AA administration alone, the AA level in eye tissues (17). If DHA uptake into tissues is faster than that of AA, and if intracellular DHA is as rapidly reduced in vivo as in cultured cells, then the use of DHA to raise intracellular AA may have a potential pharmacological application.

ACKNOWLEDGEMENT

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LEGEND TO FIGURES:

Fig. 1, Time-dependent [^{14}C]-AA uptake: At the specified times, the cells were washed and extracted for determination of radioactivity as described in the text. The uptake rate appeared to be linear for the first 30-60 min.

Fig. 2. Concentration dependent ascorbic acid (AA) uptake: The cells were incubated in complete culture medium with specified concentrations of [^{14}C]-AA at 37°C for 30 min. The cells were washed and extracted by NaOH to determinate radioactivity taken up into the cells (see text for details). Symbols represent individual determinations; data are plotted on double-reciprocal axes (Lineweaver-Burk plot).

Fig. 3. Sodium dependent AA uptake: [^{14}C]-ascorbic acid (AA), 0.34 mmol/L, was incubated with specified concentrations of NaCl for 20 min. at 37 °C temperature. The cells were washed and extracted for determination of radioactivity as described in the text. Solid line connects the average of each set of determinations.

Fig. 4. Concentration dependent DHA (DHA) uptake: The cells were incubated in complete culture medium containing different concentrations of DHA for 30 min. The cells were washed, then extracted by methanol and the AA concentration in the methanol extract was determined by high pressure liquid chromatography as described in the text. Data plotted on double-reciprocal axes.

Fig. 5. Chromatographic detection of AA and DHA in cell extract after incubation of [^{14}C]-AA with cells. (A) The cells were incubated with [^{14}C]-AA (0.34 mmol/L) and DHA (5 mmol/L) for 60 min at 37 °C. At the end of incubation, the medium was removed, the cells were rinsed with culture medium, and 50 µl methanol was added to each well to extract AA from the cells. The extract from 5 wells were combined, and 20 µl was injected into the column and eluted as described in the text. (B) Same as A except that DHA acid was omitted. (C) In order to identify the retention time of AA, the same amount of [^{14}C]-AA was injected into the column without being first incubated with the cells.

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Table 1. Kinetic properties of ascorbate and dehydroascorbate uptake:

Substrate	K_m, mmol/L	V_{max}, pmol/min/well
DHA	5.670 ± 0.290	325.50±8.4
AA	0.041 ± 0.003	2.74±0.1

Notes:

1. Average protein per well was 0.15 ± 0.01 mg/well.

2. Average cell counts per well was 2.3 x10^5.

Table 2. Effect of unlabeled dehydroascorbic acid on the uptake of ^{14}C-ascorbic acid.

AA and DHA in culture medium			
^{14}C-Ascorbate,mM	Dehydroascorbate,mM	N	^{14}C-ascorbate uptake,pmole/min/well
0.08	none	4	1.00 ± 0.12
0.08	10	4	1.24 ± 0.16
0.16	none	4	1.84 ± 0.15
0.16	10	4	1.76 ± 0.55

Table 3. Effect of sodium on DHA uptake

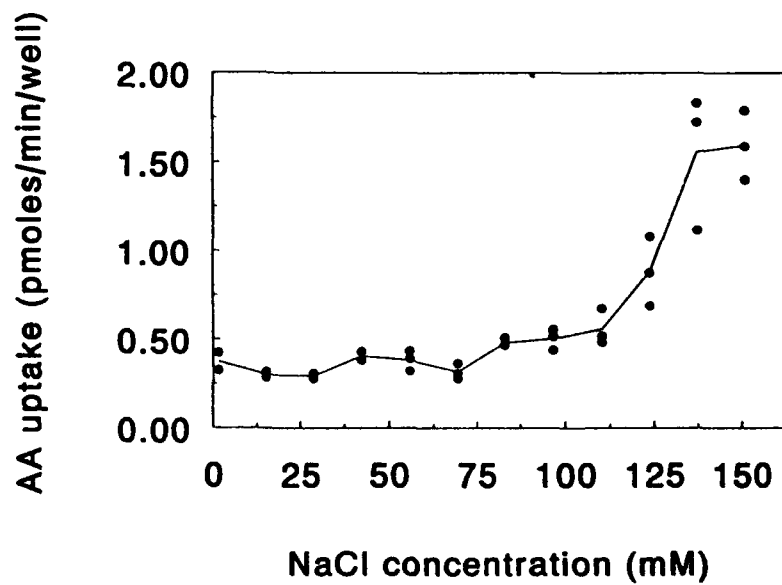| Sodium concentration	N	DHA concentration	Uptake,pmoles/min/well
30.2 mM	4	5 mM	0 ± 0.0
150.0 mM	5	5 mM	220 ± 4.0
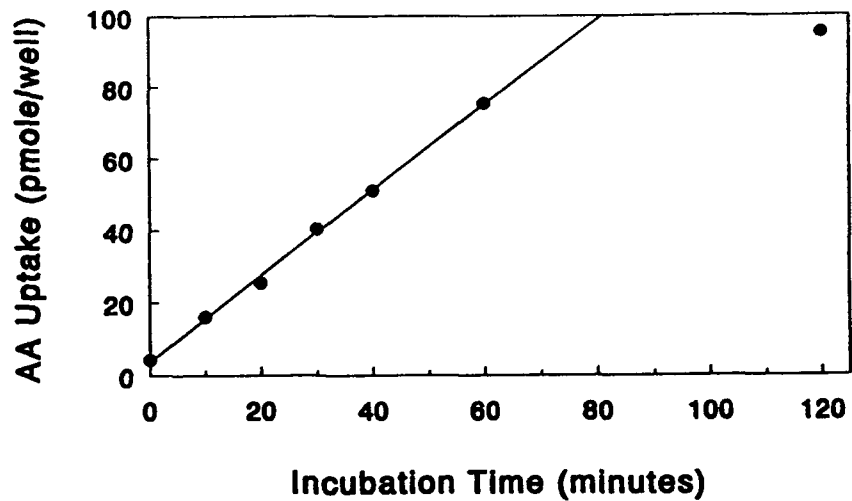
Fig. Three
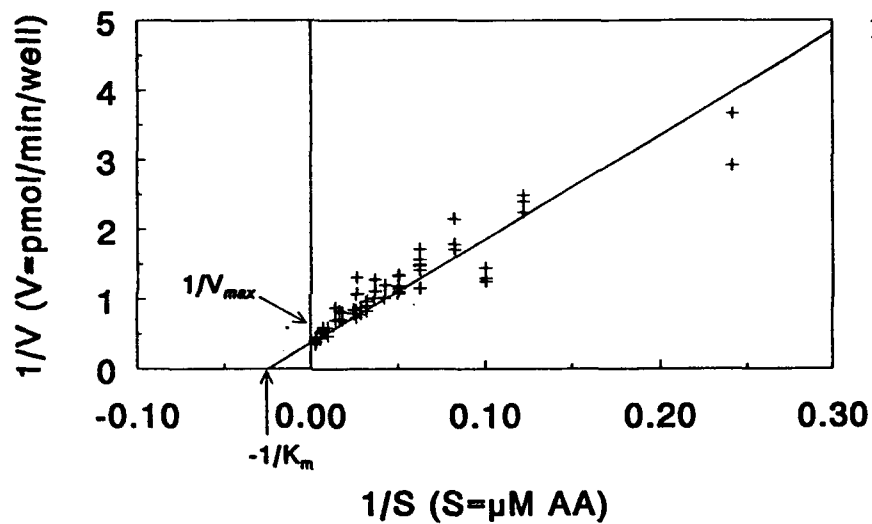
Fig. Four

Fig. Five

Fig. One

Fig. Two